Targeted Expression of Insulin-Like Growth Factor to Human Keratinocytes: Modification of the Autocrine Control of Keratinocyte Proliferation

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Somatomedin C/insulin-like growth factor-I (IGF-I) is required for the proliferation of keratinocytes *in vitro*. In skin, the cells known to synthesize IGF-I are melanocytes and fibroblasts of the dermis. To investigate the role of IGF-I as a mediator of keratinocyte proliferation, we have used retroviral-mediated gene transfer to introduce the gene encoding human IGF-I into diploid human keratinocytes, thus causing these cells to produce a growth factor they normally do not express. Modified cells synthesized and secreted significant levels of IGF-I (560 ng/10^7 cells/24 h) *in vitro*. Cells expressing IGF-I were no longer dependent on exogenously added IGF-I or insulin for their sustained growth *in vitro* under serum-free conditions. The growth of these cells did require added epidermal growth factor (EGF) and bovine pituitary extract. The addition of an antibody that neutralizes IGF-I inhibited cell growth, suggesting that IGF-I must be secreted by the cells to promote cell proliferation. To investigate the role of IGF-I *in vivo*, we grafted modified keratinocytes expressing IGF-I onto athymic mice. Grafts of epithelial sheets of modified cells formed a stratified epithelium comparable to control grafts of unmodified cells. When analyzed for keratin 16 expression and by quantitative staining for the nuclear proliferation antigen Ki-67, however, modified epithelia showed an increase in these markers of proliferation when compared with grafts of unmodified cells. This study demonstrates that genetic modification can be used to modify the autocrine control of keratinocyte proliferation. The de novo synthesis of IGF-I by keratinocytes could sustain keratinocytes growth *in vitro* and stimulate proliferation *in vivo* without significantly altering epidermal differentiation. These data further support the role of IGF-I as a paracrine mediator of epidermal proliferation and as a potential signal of mesenchymal-epithelial interactions. *Key words: gene therapy/skin grafts. J Invest Dermatol 107:113–120, 1996*

IGF-I, a single chain polypeptide of 70 amino acids, is a member of the insulin-related proteins, which include IGF-II, insulin, and relaxin (Rechler and Nissley, 1991). Unlike insulin, which regulates metabolic processes such as glucose transport, glycogen, and fat biosynthesis, IGF-I functions predominantly as a mitogenic factor and a regulator of differentiation (Kamalati *et al*, 1989; Lammers *et al*, 1989). Significant levels of IGF-I are found in the plasma, and liver production of IGF-I is stimulated by growth hormone. In fact, IGF-I is one of the primary mediators of growth hormone action (Mathews *et al*, 1986). Many other tissues produce IGF-I and it is thought that IGF-I may also serve as an autocrine/paracrine regulator of local tissue events (D'Ercole *et al*, 1984). In the skin, IGF-I is produced by melanocytes and fibroblasts but is not produced by keratinocytes, suggesting that IGF-I may be a paracrine regulator of keratinocyte proliferation (Barreca *et al*, 1992; Tavakkol *et al*, 1992).

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Abbreviations: IGF-I, insulin-like growth factor; IGFBP, insulin-like growth factor-I binding protein; EGF, epidermal growth factor; TGF-α, transforming growth factor-α.

The biologic actions of IGF-I are mediated by high affinity binding to a specific cell surface receptor (Rechler and Nissley, 1991). IGF-I binds to the specific cell surface receptor with an affinity 2–3 times higher than IGF-II and approximately 100 times higher than insulin. Conversely, IGF-I binds with low affinity to the receptor for IGF-II and insulin. In the epidermis, the receptor for IGF-I is expressed predominantly by basal keratinocytes (Krane *et al*, 1991). In the hyperproliferative disorder psoriasis, expression of IGF-I receptors is increased and is also found in suprabasal keratinocytes (Krane *et al*, 1992).

In vitro studies with serum-free cultures have demonstrated that IGF-I is one of a few growth factors required for the proliferation of keratinocytes. Insulin can substitute for IGF-I but only at concentrations that are approximately 100-fold higher than IGF-I (Tavakkol *et al*, 1992). Part of IGF-I's action may be explained by it's ability to upregulate receptors for EGF, and it's thought that this effect may increase the responsiveness of the cell to EGF and transforming growth factor-α (TGF-α), a growth factor endogenously produced by keratinocytes (Krane *et al*, 1991, 1992). Neither IGF-I nor any other growth factor alone is sufficient, however, to promote keratinocyte clonal cell growth (Wille *et al*, 1984). IGF-I is only effective *in vitro* when combined with added EGF or bovine pituitary extract (BPE) (Wille *et al*, 1984).

In this study, we used a gene transfer technology to target the expression of IGF-I to human keratinocytes to convert the action of

IGF-I from paracrine to autocrine and to study the effects of this new autocrine control of keratinocyte proliferation *in vitro* as well as *in vivo*. Keratinocytes expressing IGF-I were no longer dependent on exogenously added IGF-I or insulin for cell growth *in vitro* and formed a normal but slightly hyperproliferative epidermis *in vivo*. This approach provides additional data supporting IGF-I's role as an important mediator of human epidermal proliferation and can be used to elucidate the role of other cytokines in the skin.

MATERIALS AND METHODS

Construction of Recombinant Retrovirus A cDNA encoding human IGF-I was inserted into the *Nco*I and *Bam*HI site of the MFG retroviral vector by a three-part ligation. MFG vector DNA was digested with *Nco*I and *Bam*HI and gel purified. Vector DNA was ligated to two annealed oligonucleotides encoding the first 12 amino acids of IGF-I (5′-CATGCACACCATGTCCTCCTCGCATCTCTTCTA-3′ and 5′-CCAGGTAGAAGAGATGCGAGGAGGACATGGTGTG-3′) and a PCR product encoding the remainder of the IGF-I open reading frame. The PCR product was synthesized using 5′-CATCAGCCTGGCGCTGTGCCTGCTC-3′ as a forward primer and 5′-AGTCCAGGATCCTCCTACATCCTGTAGTTCTT-3′ as a reverse primer. After PCR, the primers were removed and the product digested with *Eco*RII and *Bam*HI and gel purified before the three-part ligation. To generate a virus-producing cell line, MFG-IGF-I plasmid DNA was transfected into a Ψ-CRIP packaging cell line (provided by R. Mulligan, Whitehead Institute of Biomedical Research, Cambridge, MA) as described in Danos and Mulligan (1988). Clones of transfectants were isolated and screened for one producing the highest titer.

Keratinocyte Cell Culture and Viral Infection Normal human keratinocytes derived from neonatal foreskins (strains A-H) were cultured using a fibroblast feeder layer as previously described using the method of Rheinwald and Green (1975). Swiss mouse 3T3-J2 feeder cells (kindly provided by H. Green, Harvard Medical School) and virus-producing cells were routinely passaged in Dulbecco's modified Eagle's medium (high glucose) (Gibco BRL, Gaithersburg, MD), supplemented with 10% bovine calf serum (HyClone, Logan, UT) and penicillin-streptomycin (100 IU per ml to 100 μg per ml, Boehringer Mannheim, Indianapolis, IN).

Keratinocytes were genetically modified as previously described (Morgan *et al*, 1987). Briefly, preconfluent primary cultures were dissociated and cells were passed onto mitomycin C-treated virus-producing cells. Four to six days after co-cultivation, modified cells were dissociated and parallel cultures were prepared for protein analysis, DNA/RNA isolation, grafting, and plating efficiency. Unmodified control cells were cultured on 3T3-J2 cells.

Measurement of Modified Keratinocyte Proliferation Rates To adjust keratinocytes to serum-free culture conditions, secondary cultures of modified and unmodified cells were washed in serum-free medium and subcultured (4×10^6 cells/10 cm dish) in keratinocyte basal medium (KBM, Clonetics) supplemented with hydrocortisone (0.5 μg per ml), bovine pituitary extract (BPE, 0.4%), epidermal growth factor (EGF, 0.1 ng per ml), insulin (5 μg per ml), and gentamicin-amphotericin-B (50 μg per ml to 50 ng per ml) (MCDB 153, Clonetics, San Diego, CA). After 2 d, cells were trypsinized, washed in serum-free medium and seeded at 10^5 cells/well per 12-well plate or at 2×10^3 cells/well per 96-well plate. Cells on 12-well plates were grown in KBM, supplemented with hydrocortisone, BPE, EGF as above, in the presence or absence of insulin (5 μg per ml) or recombinant human IGF-I (rhIGF-I) (25 ng per ml) (Boehringer Mannheim). To determine colony formation and cell proliferation, cells were formalin fixed at days 3, 6, 9, and 12 and stained with Rhodamine B (1%). To determine if IGF-I must be secreted and released from the cells to promote growth, we tested the effects of a neutralizing antibody to IGF-I. Unmodified and modified cells on 96-well plates were grown in KBM, supplemented with hydrocortisone, BPE, EGF as above, with or without rhIGF-I (40 ng per ml), respectively. Cells were grown with or without a neutralizing antibody to IGF-I (100 μg per ml, purified mouse monoclonal anti-human IGF-I antibody) (Upstate Biotechnology Inc, Lake Placid, NY) or an unrelated control rat monoclonal antibody (100 μg per ml). At days 3 and 9 the cell density was determined using a sulforhodamine B colorimetric assay. Cells were washed with $MgCl_2$ (1 mM), fixed in trichloroacetic acid (10%) (30 min, 4°C), stained with sulforhodamine B (0.4% in glacial acetic acid) (15 min, room temperature), and washed with Tris buffer (1 mM, pH 7.2). The intracellular dye was solubilized with Tris buffer (10 mM, pH 7.2) and the optical density at 570 nm was determined (THERMOmax plate reader, Molecular Devices, Menlo Park, CA).

Measurement of IGF-I Production by Modified Keratinocytes Modified keratinocytes (10^6) were plated in a 10-cm dish (containing 10^6 3T3 J-2 feeder cells) and grown to confluence. Fresh medium was added (30 ml KGM) and 1-ml samples of the culture medium were removed over a 4-d period. Samples were briefly centrifuged at 15,000 × *g* and the supernatant was assayed for IGF-I by radioimmune assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) with a sensitivity of 0.3 ng per ml. Conditioned medium was not treated by ethanol extraction, such that only free IGF-I, not complexed with IGFBPs, was detected. Cells were trypsinized and counted, and protein production was normalized to cell number. Medium conditioned by unmodified keratinocytes was used as a control.

Southern and Northern Blot Analysis Total genomic DNA was isolated from cultures of modified and control keratinocytes, digested with *Kpn*I, fractionated by gel electropheresis, transferred to a nylon filter, and analyzed by Southern blot hybridization using ^{32}P-labeled IGF-I probe. As a size standard and an estimate of the efficiency of gene transfer, 1.5, 15, and 150 pg of MFG-IGF-I plasmid DNA were digested along with 10 μg of control DNA and included on the Southern blot.

Total cellular RNA isolated from cultures of modified and control keratinocytes by the guanidium isothiocyanate method was fractionated by electrophoresis in a denaturing formaldehyde-agarose gel, blotted onto a nitrocellulose filter, and hybridized with a ^{32}P-labeled IGF-I probe.

Grafting of Keratinocyte Sheets Cultures of confluent keratinocytes (35 mm) (strain E-H) were washed twice with serum-free Dulbecco's modified Eagle's medium, detached as an intact epithelium with Dispase II solution (1.2 U per ml, 1 h, 37°C) (Boehringer Mannheim) and grafted onto anesthetized 6-to 8-week-old athymic mice (National Institutes of Health Swiss *Nu/nu*, Taconic Farms, Germantown, NY) as described by Barrandon and Green (1988). Briefly, the detached epithelium was placed basal side up on a small piece of Silastic membrane (0.005 inches thick) (Dow Corning, MI) and inserted under a full-thickness skin flap on the back of the mouse so that when the flap was closed, the basal side of the epithelium came in contact with the inner side of the mouse skin.

Histology Seven, twenty, or twenty-eight days after grafting, the human epithelium and subjacent mouse tissue were harvested for histologic analyses. The size of the graft was measured by tracing its outline on the Silastic membrane and the tissue was either fixed in formalin or immediately frozen and stored at −70°C until cryosectioned. Paraffin-embedded sections (5 μm) were stained with hematoxylin-eosin. Histologic analyses from hematoxylin-eosin stained grafts were made for control (day 7: n = 11, day 28: n = 2) and modified (day 7: n = 13, day 28: n = 2) grafts from a total of 95 mm (day 7), 6 mm (day 28) and 116 mm (day 7), 14 mm (day 28) linear length of graft, respectively.

Immunohistochemistry Cryostat sections (6 μm) fixed in acetone for 5 min and treated with blocking solution (3% bovine serum albumin, 1% normal goat serum, in phosphate-buffered saline) for 1 h at room temperature were incubated with a rabbit anti-human Ki-67 antibody (1:100) (DAKO, Glostrup, Denmark) for 30 min at room temperature (control—day 7: n = 2, day 20: n = 2; MFG-IGF-I—day 7: n = 2, day 20: n = 2; MFG-PDGF-A—day 7: n = 2 strains A-C) or with mouse monoclonal antibodies to keratins 14, 16 (kindly provided by Dr. Irene Leigh, London Hospital Medical College, London, England) or keratin 10 (1:100) (Chemicon International Inc., Temecula, CA) for 1 h at room temperature (for each keratin marker we stained control—day 7: n = 2, day 20: n = 2; MFG-IGF-I—day 7: n = 2, day 20: n = 2, strains A-C). To minimize background staining, sections were incubated with an affinity purified Fab fragment of goat anti-mouse IgG (1:100) (Jackson ImmunoResearch Laboratories, West Grove, PA) prior to staining with any of the mouse monoclonal primary antibodies. Slides were washed 4 × 15 min in phosphate-buffered saline, then incubated with fluorescein-conjugated affinity purified goat anti-mouse IgG or IgM antibody (1:50) or goat anti-rabbit IgG antibody (1:100) (Jackson ImmunoResearch Laboratories) for 1 h room temperature. The slides were washed 4 × 15 min in phosphate-buffered saline and coverslipped using 1% n-propyl-gallate mounting solution (Sigma, St. Louis, MO). Antibodies were diluted in blocking solution and control sections were not exposed to primary antibody.

Assessment of Epidermal Proliferation The proliferative activity of keratinocytes in modified and unmodified epithelia and neonatal foreskins was assessed by staining with a monoclonal antibody to Ki-67, a nuclear cell proliferation-associated antigen (Ando *et al*, 1990; Hofman-Wellenhof *et al*, 1990). Ki-67–positive cells, located in the basal or immediately suprabasal layer, were counted from the following lengths of grafts: 17.1 mm (day 7) and 11.7 mm (day 20) for control grafts; 29.7 mm (day 7) and 8.1 mm (day 20) for IGF-I–modified grafts; 14.4 mm (day 7) for PDGF-A–modified grafts; and 28.3 mm for foreskin samples; these were presented as a

percentage of the total number of basal cells. Hyperproliferative edges of epithelial grafts were excluded. Statistical comparisons were performed using the Student's t test for unpaired samples.

RESULTS

Genetic Modification of Human Keratinocytes The structure of the retroviral vector used to express the IGF-I gene in human keratinocytes is shown in **Fig 1*A***. A cDNA encoding human IGF-I was inserted into the MFG vector, which was then transfected into the Ψ-CRIP packaging cell line. A clone of the Ψ-CRIP packaging cell line producing the MFG-IGF-I recombinant virus was isolated. Recombinant viruses produced by the Ψ-CRIP cells are free of detectable replication-competent virus and have an amphotropic host range.

Secondary cultures of human keratinocytes established from neonatal foreskins were co-cultivated with mitomycin C-treated Ψ-CRIP cells producing the MFG-IGF-I virus. After 4 d of cocultivation, the virus-producing cells were removed, and the keratinocytes were dissociated using trypsin and passed onto a normal 3T3 fibroblast feeder layer.

The MFG-IGF-I recombinant genome was detected in the cellular DNA of modified keratinocytes by Southern blot hybridization. Genomic DNA prepared from modified and control keratinocytes was cleaved with *Kpn*I and transferred to a nylon membrane after fractionation by gel electrophoresis. Because *Kpn*I cleaves three times in the MFG-IGF-I recombinant vector, once in each LTR and once upstream of the IGF-I insert, it excises a 1226 base pair fragment containing the IGF-I cDNA. Hybridization with ^{32}P-labeled IGF-I gene sequences revealed the presence of the MFG-IGF-I fragment of the correct size in the cellular DNA of modified cells **(Fig 1*B*)**. When compared with the hybridization signal from 1.5, 15, and 150 pg of MFG-IGF-I plasmid DNA, it is clear that the modified cells contain multiple copies of the IGF-1 gene.

Genetically Modified Human Keratinocytes Secrete IGF-I Transcription of the MFG-IGF-I provirus was verified by northern-blot hybridization of total RNA isolated from control and MFG-IGF-I–transduced cells **(Fig 1*C*)**. After hybridization with ^{32}P-labeled IGF-I gene sequences, the expected transcripts from the MFG-IGF-I provirus were detected in RNA isolated from modified cells. In accordance with previous findings, endogenous IGF-I mRNA was not detected in unmodified keratinocytes (Barreca *et al*, 1992; Tavakkol *et al*, 1992).

To measure the production of IGF-I by modified keratinocytes, cells were grown to confluence, fresh medium was added (30 ml), and portions (1 ml) of the culture medium were removed over a 4-d period. The level of IGF-I was measured by radioimmunoassay. IGF-I was continuously secreted by modified keratinocytes up to 4 d with an average daily secretion rate of 560 ng/10^7 cells/24 h **(Fig 2)**. IGF-I produced by modified cells was readily detected without ethanol extraction of conditioned medium indicating that significant quantities of IGF-I are free and not bound to IGF-binding proteins (IGFBPs). Medium conditioned by unmodified keratinocytes did not contain any IGF-I (data not shown).

Expression of IGF-I Modifies the Autocrine Control of Keratinocyte Proliferation The growth of IGF-I–modified keratinocytes was determined in serum-free cultures, where the role of various additives is well defined. Optimal keratinocyte growth in MCDB 153 medium requires added EGF, hydrocortisone, BPE, and insulin. If either EGF or insulin are omitted the cells will not grow. The effects of insulin are thought to be partially mediated through the IGF-I receptor because insulin binds this receptor with low affinity, and a monoclonal antibody to the IGF-I receptor can partially block the growth promoting activity of insulin (Neely *et al*, 1991). Moreover, IGF-I can substitute for insulin in the serum-free culture of keratinocytes (Neely *et al*, 1991). IGF-I alone is not sufficient for keratinocyte growth and optimal growth requires added EGF and BPE.

Unmodified and IGF-I-expressing cells were cultured in serum-free medium with or without insulin or rhIGF-I. At days 3, 6, 9, and 12, the cells were fixed and stained **(Fig 3)**. Unmodified keratinocytes showed little or no growth in the absence of insulin or rhIGF-I. As an additional control for the effects of genetic modification, we tested cells that were modified to express PDGF-A, a

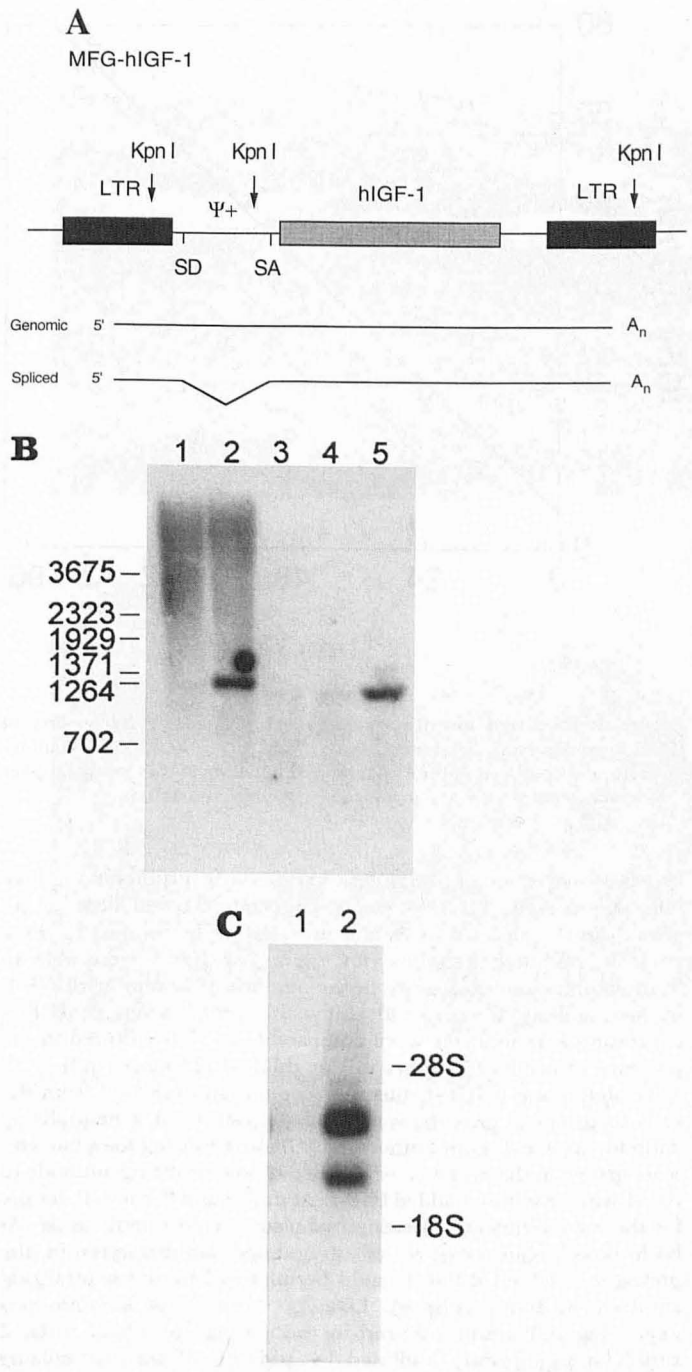

Figure 1. Integration and transcription of recombinant retrovirus. *A*, proviral structure of MFG-IGF-I recombinant retrovirus and expected RNAs are diagrammed. Shown is the LTR, long terminal repeat; SD, splice donor; SA, splice acceptor; Ψ +, packaging signal and sites of digestion with Kpn1. *B*, Southern blot of cellular DNA (10 μg) digested with *Kpn*I and probed by hybridization to a [^{32}P]IGF-I gene sequences. Genomic DNA isolated from control keratinocytes (unmodified) (*lane 1*) and MFG-IGF-I–modified keratinocytes (*lane 2*). Control DNA mixed with MFG-IGF-I plasmid DNA 1.5, 15, and 150 pg (*lanes 3*, *4*, and *5*, respectively). Shown are size markers. *C*, northern blot of total RNA (5 μg) isolated from control (*lane 1*) and MFG-IGF-I–transduced keratinocytes (*lane 2*). Filter was hybridized with a [^{32}P]IGF-I probe. Shown are the rRNA markers.

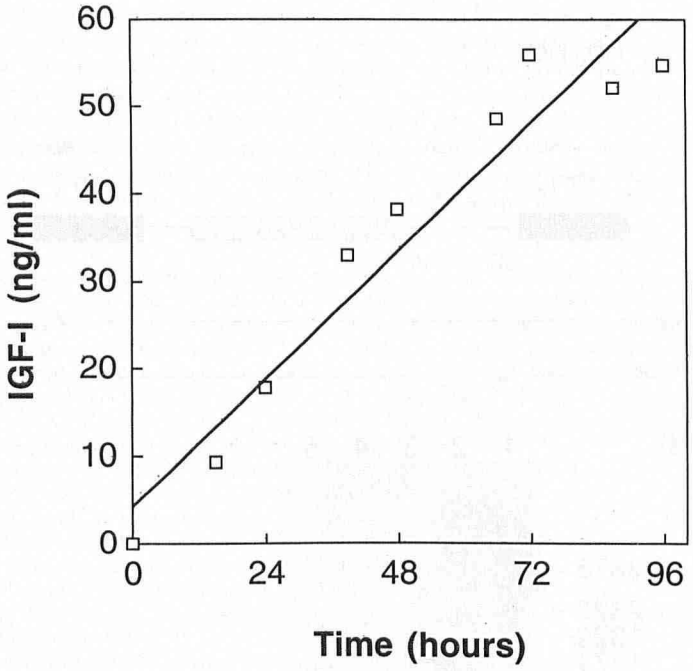

Figure 2. Modified keratinocytes secrete IGF-I. A time course of IGF-I secretion was determined by removing portions of the culture medium of a confluent culture of modified keratinocytes as indicated over a 4-d period and, IGF-I was measured by radioimmunoassay.

cytokine that does not stimulate keratinocyte proliferation. Like unmodified cells, PDGF-A-expressing cells showed little or no growth in the absence of insulin or rhIGF-I. In contrast to these controls, modified keratinocytes expressing IGF-I were able to form colonies and proliferate in the absence of insulin or rhIGF-I. In fact, colony forming efficiency and proliferation of IGF-I-expressing keratinocytes were comparable to cells cultured in the presence of insulin (5 μg per ml) or rhIGF-I (25 ng per ml).

To determine if IGF-I must be secreted and released from the cells to promote growth, we tested the effects of a neutralizing antibody to IGF-I. Unmodified and IGF-I-expressing keratinocytes were grown in the presence or absence of a neutralizing antibody to IGF-I with or without added IGF-I. At days 3 and 9, the cell density for these conditions was determined using a colorimetric assay. At both time points, growth of unmodified keratinocytes in the presence of added rhIGF-I could be inhibited by the neutralizing antibody to IGF-I **(Fig 4)**. Likewise, growth of keratinocytes expressing IGF-I and cultured in the absence of added rhIGF-I could be significantly inhibited by addition of the neutralizing antibody **(Fig 4)**. These data further confirm that the new autocrine growth control of the modified cells is mediated by IGF-I and that because its action can be inhibited by a neutralizing antibody, IGF-I must be secreted to promote growth. Exposure of cells to an unrelated control rat monoclonal antibody did not affect cell growth (data not shown).

Keratinocytes Expressing IGF-I Form a Stratified Epithelium When Transplanted to Athymic Mice Keratinocytes secreting IGF-I were tested for their ability to form an epidermis when transplanted to athymic mice. Normal or modified keratinocytes were grown to confluence in a 35-mm dish. The cells were treated with Dispase, detached as an epithelial sheet, and grafted onto the underside of a dorsal skin flap. The basal side of the sheet was placed directly on the epimysium of the mouse panniculus carnosus. Seven and twenty-eight days after grafting the human epithelium and the subjacent mouse tissue, the sheets were fixed and stained.

Both unmodified cells and cells expressing IGF-I developed a

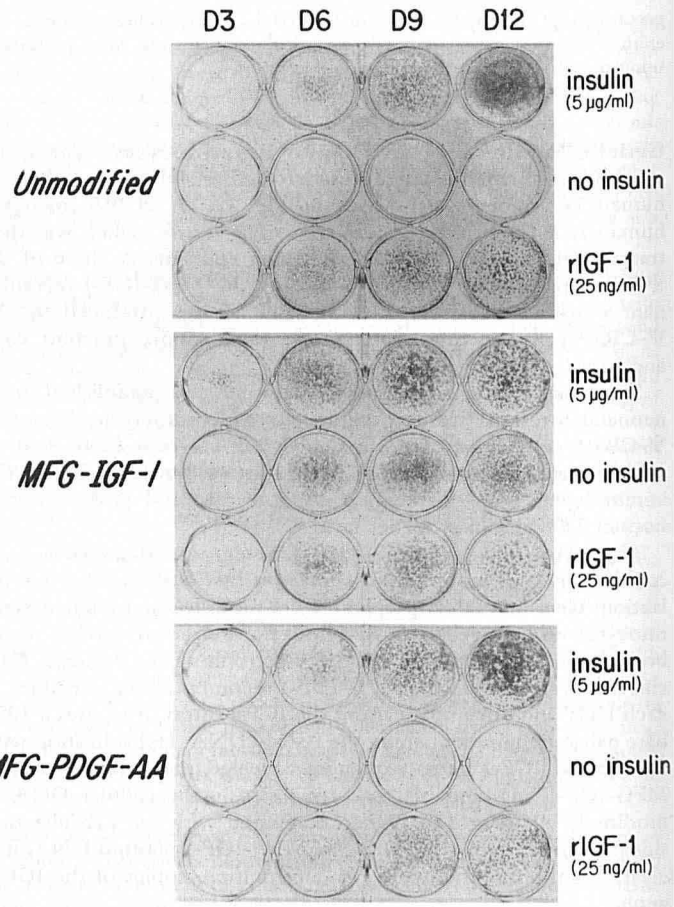

Figure 3. Expression of IGF-I by keratinocytes modifies the autocrine control of keratinocyte proliferation. Unmodified, IGF-I, or PDGF-A–modified keratinocytes were grown in serum-free medium, with or without insulin or rhIGF-I. At indicated time points cultures were fixed and stained with Rhodamine B.

stratified epidermal structure consisting of stratum basale, spinosum, granulosum, and corneum, indicating complete terminal differentiation. Seven days after grafting, the epithelia of modified and unmodified cells were several cell layers thick **(Fig 5*A*,*B*)**. Twenty-eight days after grafting, the thickness of both epithelia

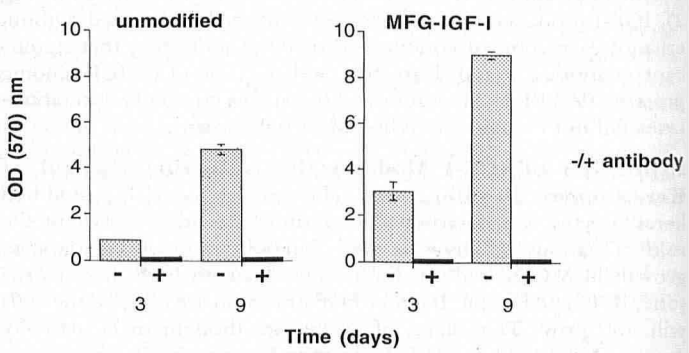

Figure 4. Bioactivity of IGF-I released from modified keratinocytes can be blocked by an IGF-I neutralizing antibody. Unmodified and MFG-IGF-I-modified keratinocytes were grown in serum-free medium in the presence or absence of rhIGF-I (40 ng per ml). To block the effect of IGF-I on growth, cells were grown in the presence or absence of a neutralizing antibody to IGF-I. At indicated time points cells were fixed and cell numbers determined using a colorimetric assay.

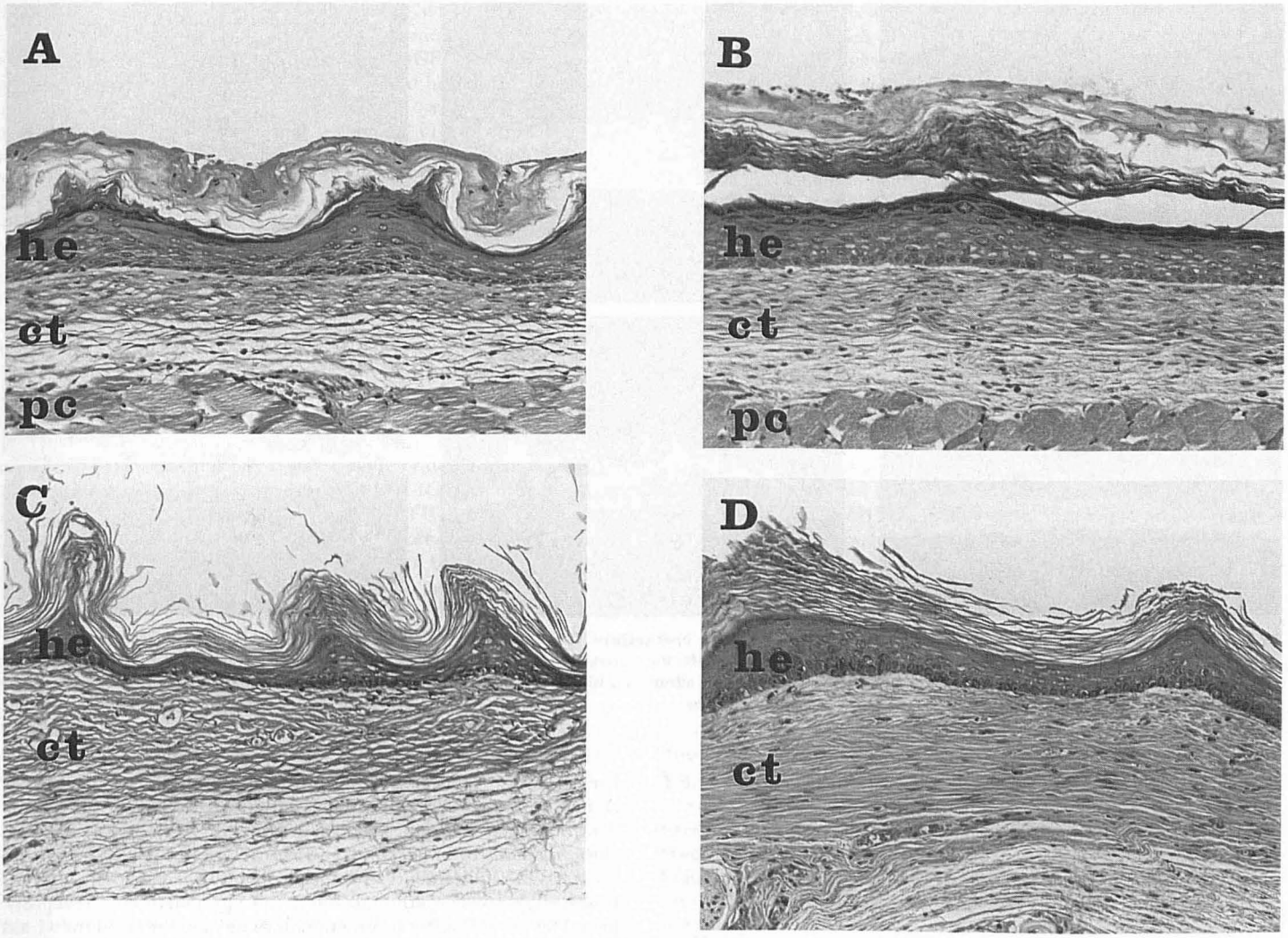

Figure 5. IGF-I-secreting keratinocytes form a stratified epithelium when transplanted to athymic mice. Cultures of confluent epithelia generated from unmodified (*A*, *C*) or MFG-IGF-I-modified keratinocytes (*B*, *D*) were detached from the dish with Dispase treatment and grafted under a full thickness skin flap of athymic mice. Seven (*A*, *B*) and 28 (*C*, *D*) days later the grafts were fixed, and paraffin-embedded sections (5 μm) were stained with hematoxylin and eosin. *he*, human epidermis; *ct*, connective tissue; *pc*, panniculus carnosus (*bar*, 100 μm).

were diminished with fewer cells in the stratum spinosum and granulosum, compared with day 7 after grafting **(Fig 5*C,D*)**. Overall, the morphologies of the epidermis formed by control and IGF-I-expressing keratinocytes were indistinguishable at both time points. In this grafting model, the epithelial graft is placed subdermally and the cornified layer does not exfoliate; thus, the thickness of the stratum corneum increases with time.

The connective tissues that formed subjacent to grafts of IGF-I-expressing cells and unmodified cells were also similar. At day 7, the tissue subjacent to control and IGF-I-expressing grafts was a loose connective tissue, containing fibroblasts and occasional mononuclear cells. Within this connective tissue were uniformly distributed fibers oriented parallel to the overlying epidermis. At day 28, the connective tissue adjacent to modified and unmodified epithelia decreased slightly in cellularity and the collagen fibers appeared thicker and more dense, compared with day 7. No difference in cellularity, vascularity, or thickness of the connective tissue was evident between modified and unmodified grafts. These results are in contrast to our previous data with grafts expressing PDGF-A in which the subjacent connective tissue was significantly thicker, more cellular, and highly vascularized compared with control grafts (Eming *et al*, 1995).

Markers of Proliferation Are Elevated in IGF-I-Expressing Grafts To determine whether keratin expression was altered in grafts of IGF-I-expressing keratinocytes, we stained sections of modified and unmodified epithelia, as well as normal neonatal foreskin with monoclonal antibodies specific for keratins 1/10 (K1/10), 14 (K14), and 16 (K16). In normal epidermis, K14 is synthesized in the basal layer (Purkis *et al*, 1990) and K1/10 are expressed in suprabasal layers (Weiss *et al*, 1984). In hyperproliferative skin diseases such as psoriasis, K14 expression often extends to the suprabasal layers, and a new keratin, K16, is induced in the differentiating cells (Weiss *et al*, 1984).

In the epidermis of the neonatal foreskin, staining for K14 was confined exclusively to the basal layer, whereas staining for K1 and K10 was observed only in suprabasal layers, and no staining for K16 was evident (data not shown). Seven days after grafting, modified and unmodified epithelia showed comparable strong staining for K14 throughout the entire epithelium **(Fig 6*A–B*)** and staining for K10 in the suprabasal layers **(Fig 6*C–D*)**. In contrast, K16 staining was weak in suprabasal layers of control grafts and strong in IGF-I–modified grafts **(Fig 6*E,F*)**. Staining for K14, K10, and K16 at day 20 for control and modified epithelia was decreased when compared with day 7 after grafting. Suprabasal staining for K16 in IGF-I–modified grafts remained stronger, however, compared with unmodified grafts at this time (data not shown). Collectively, the keratin staining pattern at 7 and 20 d postgrafting by modified and unmodified epithelia were similar to those described for a regen-

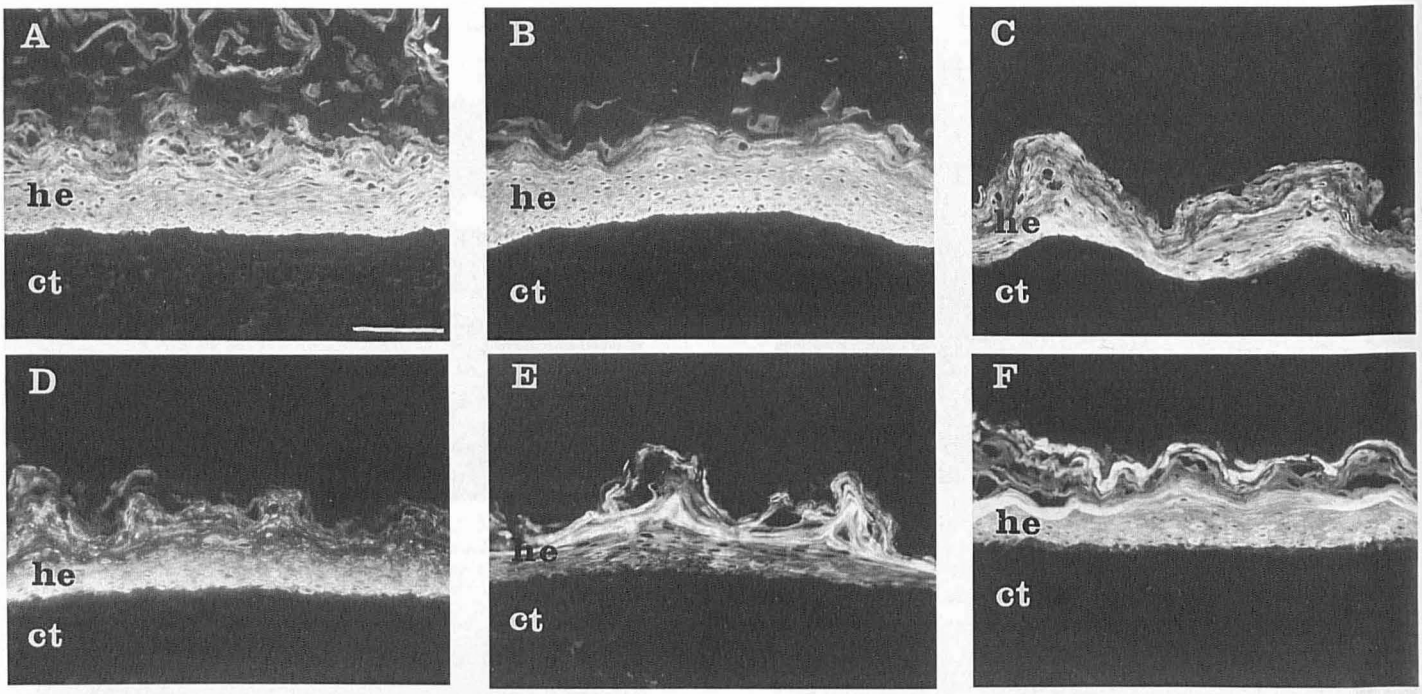

Figure 6. K16 expression is elevated in IGF-I–overexpressing epithelium. Unmodified (*A*, *C*, *E*) and IGF-I-modified (*B*, *D*, *F*) epithelia were transplanted to athymic mice. At day 7, grafts were frozen, and cryosections were stained using mouse monoclonal antibodies to K14 (*A*, *B*), K10 (*C*, *D*), and K16 (*E*, *F*). Staining was visualized with a fluorescein-conjugated affinity-purified goat-anti-mouse IgG antibody. *he*, human epidermis; *ct*, connective tissue (*bar*, 100 μm).

erative epidermis. Compared with controls, the effects of IGF-I expression were evident only as an increase in K16 expression.

As a further test for proliferation *in vivo*, we stained tissue sections from biopsies at 7 and 20 d after grafting, with a monoclonal antibody to Ki-67, a nuclear antigen associated with cell proliferation. The Ki-67 antigen is present in the nuclei of cells in late G1, S, G2, and M phases, but is absent from the G0 and the early G1 phases (Ando *et al*, 1990; Hofman-Wellenhof *et al*, 1990). Epithelia derived from IGF-I-expressing cells had significantly increased numbers of Ki-67–positive cells at 7 and 20 d after grafting compared with grafts of unmodified cells, grafts of cells expressing PDGF-A, or neonatal foreskin **(Table I)**. On average, IGF-I-expressing grafts had a 2-fold increase (day 7) or a 4-fold increase (day 20) in Ki-67–positive cells, when compared with control epithelia **(Table I)**. The Ki-67–positive cells of unmodified and PDGF-A-expressing epithelia were predominantly in the basal layer, with few positive cells in the suprabasal layer. In contrast, IGF-I-expressing epithelia had increased numbers of Ki-67–positive cells in the basal layer as well as significant numbers of positive cells in the suprabasal layer.

DISCUSSION

In this study we investigated the *in vitro* and *in vivo* effects of altering the autocrine growth control of human keratinocytes. Human diploid keratinocytes were genetically modified by retroviral-mediated gene transfer to express IGF-I, an important mitogen for keratinocytes and a cytokine they normally do not express. Modified cells synthesized IGF-I at levels up to 560 ng/10^7 cells/24 h. When grown in serum-free cultures, IGF-I-expressing cells no longer required added IGF-I or insulin, thus demonstrating that genetic modification had engineered a new autocrine control for these cells. Neutralizing antibody to IGF-I inhibited the growth of the cells indicating that IGF-I autocrine control of these cells required secretion of IGF-I.

Our findings are consistent with earlier work that demonstrated the strict requirement for IGF-I or high-dose insulin for the growth of human keratinocytes in serum-free culture (Wille *et al*, 1984). These studies also showed that IGF-I alone was not sufficient for keratinocyte growth and that optimal growth required added EGF and BPE. Likewise, our IGF-I-secreting cells were also still dependent on added EGF and BPE for growth, suggesting that this new autocrine control of keratinocytes was selective for IGF-I and that the growth requirements of the cells had not been grossly altered. It also suggests that although IGF-I can upregulate EGF receptor/ligand levels, this effect is not enough to obviate the need for added EGF to promote growth (Krane *et al*, 1991; Vardy *et al*, 1995).

In many cell types, the action of IGF-I appears to be controlled

Table I. Proliferation of IGF-I-Expressing Keratinocytes *In Vivo*

Strains[a]	Day[b]	Ki67-positive cells (%) (±SEM)[c]	Length of graft (mm^2)
Control			
A	7	13.9 (±6.7)	8.1
B	7	14 (±8.2)	9.0
C #1	20	10.4 (±3.9)	5.4
C #2	20	10.7 (±3.0)	6.3
MFG-PDGF-A			
A	7	8.6 (±5.6)	5.4
B	7	10.0 (±2.8)	9.0
MFG-IGF-1			
A	7	33.9[d] (±15.3)	19.8
B	7	20.45[d] (±6.2)	9.9
C #1	20	43.75[d] (±11.3)	3.6
C #2	20	34.38[d] (±14.7)	4.5
Neonatal foreskin		11.0 (±3.6)	28.3

[a] Epidermal grafts were made of human epidermis generated from unmodified or MFG-IGF-I–transduced keratinocytes.

[b] Epidermal grafts were harvested 7 and 20 d after grafting.

[c] Activation of cellular proliferation was quantitated by the counting of keratinocytes expressing Ki67+ nuclei located in the basal or immediately suprabasal layer and presented as a percentage of basal cells per field of view.

[d] $p < 0.05$.

by IGF-binding proteins (IGFBPs-1 to -6), which modulate the interaction of IGF-I with its receptor (Clemmons, 1992). Most *in vitro* data demonstrate that the complex of IGF-I with its binding protein is not recognized by the receptor and hence is biologically inactive. Conversely, there is evidence that certain IGF-I-binding proteins, such as IGFBP-1 and -3, have both inhibitory and stimulatory effects on IGF-I actions, depending on the experimental condition (De Mellow and Baxter, 1988; Tsuboi *et al*, 1995). In the skin, dermal fibroblasts produce IGFBPs -3, -4, -5, and -6; and recently, it has been shown that human keratinocytes synthesize IGFBPs -2, -3, -4, and -6 (Murashita *et al*, 1995). One study suggests that keratinocyte produced IGFBPs may modulate keratinocyte responsiveness to IGF-I. They showed that a mutant form of IGF-I that doesn't bind IGFBPs was a more potent mitogen for keratinocytes *in vitro* than normal IGF-I (Wraight *et al*, 1994). In our system, IGF-I produced by modified cells was readily detected without the need for ethanol extraction of conditioned medium to separate IGF-I from its IGFBPs, indicating that significant quantities of IGF-I were free and not complexed with IGFBPs. Moreover, keratinocytes producing IGF-I were no longer dependent on added IGF-I for growth *in vitro* demonstrating that IGF-I synthesized by modified cells was biologically active. Thus, if IGF-I synthesized by modified keratinocytes is complexed with keratinocyte produced IGFBPs, there is sufficient free IGF-I produced by modified cells to be detected by radioimmune assay and to act as an autocrine stimulator of keratinocyte growth.

One of the advantages of using retroviral-mediated gene transfer to genetically modify cells is that the gene is stably integrated and the effects of genetic modification can be evaluated *in vitro* as well as *in vivo* by transplantation of a transgenic epithelium. In this way, the effects of new autocrine controls on the growth and differentiation of the modified tissue can be examined, as well as the effects on adjacent neighboring tissues. Keratinocytes expressing IGF-I were grafted as an epithelial sheet to athymic mice wherein control unmodified cells and cells secreting IGF-I formed comparable stratified epithelia. Both epidermal structures were several cell layers thick complete with stratum granulosum and corneum. Slight differences were seen when the grafts were stained for keratin gene expression. Seven days after grafting, the IGF-I-secreting tissue had increased staining for keratin 16, a marker for regenerative epithelium. More dramatic was the increase in the number of proliferating cells in the IGF-I epithelium versus the control epithelia. The number of Ki-67–positive cells in the basal and suprabasal layers of IGF-I grafts was significantly increased over controls at 7 and 20 d after grafting. Nevertheless, this increase in proportion of proliferating cells did not grossly alter keratinocyte growth and differentiation *in vivo*. The increase in proliferating cells could be balanced by an increase in the rate of terminal differentiation. Thus, elevated levels of IGF-I may be necessary for an increase in the proliferation of the epidermis, but high levels of IGF-I alone are not sufficient to cause gross alterations of the epidermis as seen in the hyperproliferative disorder, psoriasis.

Transgenic mice in which cytokine overexpression has been directed to the epidermis have been used to address similar questions regarding autocrine/paracrine growth control of the epidermis. When the gene encoding TGF-α, a cytokine endogenously made by keratinocytes, was linked to a keratin promoter and overexpressed in the stratified squamous epithelia of transgenic mice, proliferation of basal keratinocytes was enhanced and epidermal thickening persisted for 2 weeks after birth (Vassar and Fuchs, 1991). When the gene encoding keratinocyte growth factor, a cytokine not produced by keratinocytes (normally produced by dermal fibroblasts), was expressed using the keratin promoter, the transgenic mice also had a thickened epidermis that persisted for 4 mo (Guo *et al*, 1993). These studies suggest that TGF-α and keratinocyte growth factor are important autocrine and paracrine mediators of epidermal homeostasis, respectively. As in our results with IGF-I, however, overexpression of neither cytokine alone was sufficient to sustain prolonged hyperproliferation of the epidermis.

The approach used in our study can serve as an alternate method for generating animals with a transgenic epithelium and can be used to ask similar questions of the human epidermis. One aspect of the need for alternate *in vivo* models is evident from two recent reports of mice with targeted disruptions of the gene encoding the receptor for EGF (Sibilia and Wagner, 1995; Threadgill *et al*, 1995). The phenotype of these EGF receptor knockout mice was highly dependent on the genetic background of the strain of mice used. On one genetic background homozygous mutants died *in utero* because of defects in the placenta, whereas mutants in a different genetic background survived for up to 3 weeks and had defects in the skin and other organ systems.

In our grafting model, a connective tissue forms adjacent to the grafted epithelium. Thus, we are able to determine not only the autocrine actions of an overexpressed cytokine, but also its paracrine effects on the adjacent tissue. In a previous report, we showed that the connective tissue subjacent to grafts overexpressing PDGF-A was significantly thicker, more cellular, and more vascular than controls 7 d after grafting (Eming *et al*, 1995). IGF-I has numerous effects on connective tissue cells *in vitro*. It stimulates proliferation of fibroblasts and their production of collagen type I and is chemotactic and mitogenic for endothelial cells (Grant *et al*, 1987; Granot *et al*, 1991; Bar *et al*, 1988). Yet, in the present study, the connective tissues formed adjacent to the control and IGF-I-expressing epithelium were comparable with no gross differences in thickness, cellularity, or vascularity.

Several factors could influence IGF-I's effect on connective tissue *in vivo*. The host supply of IGF-I in connective tissue may not be a limiting factor, or dermal- and epidermal-derived IGFBPs could interact with the keratinocyte-produced IGF-I and modulate its paracrine action on the connective tissue. Alternatively, IGF-I may not have the activities of PDGF that stimulate connective tissue formation in this model, such as PDGF's ability to be chemotactic for neutrophils, macrophages, and fibroblasts. Lastly, IGF-I may have a more prominent role in regulating the epidermis rather than the dermis. Mice with a targeted disruption of the type I IGF-I receptor have a relatively unaffected dermis, whereas epidermal development and hair morphogenesis were severely affected with few spinous keratinocytes and premature epidermal differentiation (Liu *et al*, 1993).

IGF-I may be useful for cutaneous tissue repair. IGF-I has been detected in human wound fluid and its expression is elevated in granulation tissue after injury of the skin (Steenfos and Jansson, 1992). Topical application of recombinant IGF-I simultaneous with PDGF in animal studies results in accelerated cutaneous tissue repair, and systemic administration of recombinant human growth hormone to patients with severe burns elevated their reduced IGF-I serum levels and accelerated re-epithelialization of partial thickness wounds (Lynch *et al*, 1987; Gore *et al*, 1991; Gilpin *et al*, 1994). One limitation of the systemic administration of growth hormone is its diabetogenic properties. Cultured grafts expressing IGF-I may be useful for the local synthesis and delivery of IGF-I in an IGF-I-deficient condition.

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